Alternatives to Antibiotics: Utilization of Bacteriophage to Treat Colibacillosis and Prevent Foodborne Pathogens[1]

W. E. Huff,[2] G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue

Poultry Production and Product Safety Research Unit, USDA, Agricultural Research Service, Poultry Science Center, University of Arkansas, Fayetteville, Arkansas 72701

ABSTRACT Bacteriophages are viruses that infect and kill bacteria. Bacteriophage do not infect animal and plant cells, which makes them a potentially safe alternative to antibiotics. We have been conducting research on the efficacy of bacteriophage to prevent and treat colibacillosis in poultry. Bacteriophages that were lytic to a nonmotile, serotype O2 isolate of *Escherichia coli* were isolated from municipal wastewater treatment plants and poultry processing plants. This *E. coli* isolate is pathogenic to poultry, causing severe respiratory and systemic infections. Two bacteriophage isolates were selected for use in studies designed to determine the efficacy of these bacteriophage to prevent and treat severe colibacillosis in poultry. Colibacillosis was induced by injecting 6×10^4 cfu of *E. coli* into the thoracic air sac when birds were 1 wk of age. Initial studies demonstrated that mortality was significantly reduced from 85 to 35% when the challenge culture was mixed with equal titers of bacteriophage, and the birds were completely protected when the challenge culture was mixed with 10^8 pfu of bacteriophage. In subsequent studies, we have shown that an aerosol spray of bacteriophage given to birds prior to this *E. coli* challenge could significantly reduce mortality even when given 3 d prior to the *E. coli* challenge. Our research on treating colibacillosis in poultry has demonstrated that an intramuscular injection of bacteriophage given 24 or 48 h after the birds were challenged rescued the birds from this severe *E. coli* infection. We have demonstrated that bacteriophage can be used to prevent and treat colibacillosis in poultry and may provide an effective alternative to antibiotic use in animal production.

(*Key words*: bacteriophage, *Escherichia coli*, chicken)

2005 Poultry Science 84:655–659

INTRODUCTION

Bacteriophages are viruses that infect and kill bacteria. Bacteriophage kill bacteria, which provides an opportunity to use bacteriophage as an alternative to antibiotics to prevent and treat bacterial infections and to reduce foodborne pathogens on agricultural products. Bacteriophages are safe, having no activity against animal or plant cells, and appear to have evolved with bacteria as they are ubiquitous in nature. Bacteriophages were codiscovered in the early 1900s by Twort (1915) and d'Herelle (1917). Bacteriophages are typical viruses that have a protein coat that encloses a nucleic acid, which can be DNA or RNA. There are 2 general types of bacteriophage, virulent and temperate, and they differ in life cycle. Virulent bacteriophage kill bacteria through a multiple-step process. First they adsorb to bacteria through recognition of specific attachment sites (receptors) on the surface of the bacteria. Their nucleic acid (DNA or RNA) is then injected into the bacterium. Viral replication is then achieved in the bacteria. The bacteria are destroyed through lysis, resulting in an average release of 50 to 200 daughter particles. Temperate bacteriophage do not immediately replicate in the bacterium they infect, but coexist within the bacterium as a prophage (viral nucleic acid inserted into the bacterial genome), that is replicated along with the bacterium, thereby converting the bacterium to a lysogenic strain. When lysogenic bacteria are stressed, the prophage can become activated, resulting in replication of the virus and killing of the bacteria through lysis. Generally, virulent bacteriophage provides the greatest opportunity in various applications to control bacteria.

Almost immediately after their discovery, there was interest in using bacteriophage to prevent and treat bacterial infections. There were successful applications of bacteriophage therapeutics, but therapeutic efficacy of bacteriophage was inconsistent, and research and product development was for the most part discontinued with the development of antibiotics. There were exceptions to this trend mainly in Eastern Europe. The Eliava Institute (Tblisi, Georgia, of the former Soviet Union) has contin-

©2005 Poultry Science Association, Inc.

Received for publication July 1, 2004.

Accepted for publication September 16, 2004.

[1]Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

[2]To whom correspondence should be addressed: huff@uark.edu.

ued bacteriophage research to the present. The Eliava Institute was established in 1923 by Giorgi Eliava a former student of Felix d'Herelle. The Russian research on bacteriophage in human medicine was reviewed by Alisky et al. (1998). Besides the continued research on bacteriophage conducted at the Eliava Institute, there are a number of papers on work done in Poland using bacteriophage therapy in human medicine (Ślopek et al. 1981, 1984, 1985, 1987; Weber-Dąbrowska et al. 1987).

There is growing interest in the use of bacteriophage to control bacterial infections. The ability of bacteriophage to control diarrhea induced by *Escherichia coli* in calves, piglets, and lambs has been demonstrated (Smith and Huggins, 1983; Smith et al., 1987). These authors have also demonstrated the ability of phage to treat *E. coli* infections in mice (Smith and Huggins, 1982). Barrow et al. (1998) demonstrated the ability of bacteriophage to protect chickens from intramuscular challenge with *E. coli* when bacteriophage is simultaneously injected at different sites. Soothill (1992) found that bacteriophage would protect mice from infection with *Acinetobacter baumanii* and *Pseudomonas aeruginosa*. Vancomycin-resistant *Enterococcus faecium* is a serious and growing nosocomial infection. Biswas et al. (2002) demonstrated that bacteriophage could rescue mice from a lethal challenge with vancomycin-resistant *E. faecium*. Bacteriophage provides effective control of disease caused by *Pseudomonas plecoglossicida* in ayu, a fish raised in Japan (Park et al., 2000). Matsuzaki et al. (2003) was able to protect mice from a lethal injection of *Staphylococcus aureus. Vibrio vulnificus* is an opportunistic pathogen of humans that can result in septicemia and is often associated with the consumption of oysters. Cerveny et al. (2002) have demonstrated that bacteriophage had therapeutic value in the treatment of localized and systemic infections with *V. vulnificus* in a murine model.

There is significant research on the use of bacteriophage to control foodborne pathogens, such as *Salmonella*, *Listeria monocytogenes*, *E. coli* O157:H7, and *Campylobacter* on agricultural products. Research demonstrating the efficacy of bacteriophage to reduce *L. monocytogenes* on fresh-cut produce has been demonstrated by Leverentz et al. (2003). Lytic bacteriophage have been shown to decrease *Salmonella* and *Campylobacter* contamination on chicken skin (Goode et al., 2003). There is also a significant research effort being conducted on controlling *Salmonella* on poultry products (Higgins et al., 2002).

RESEARCH ON BACTERIOPHAGE TO PREVENT AND TREAT COLIBACILLOSIS

We have been conducting research on the use of bacteriophage to prevent and treat colibacillosis in broiler chickens for the past several years. We isolated bacteriophage to an *E. coli* poultry isolate (serotype 02) using either waste water from municipal sewer treatment plants or poultry processing plants as described by Huff et al. (2002b). Two bacteriophage isolates, designated SPR02

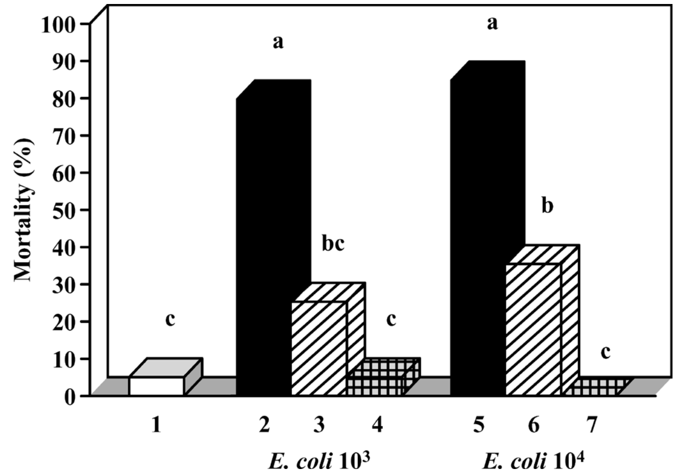

FIGURE 1. The effect on mortality (%) after mixing bacteriophage (SPR02) with an *Escherichia coli* challenge culture to prevent severe colibacillosis in broiler chickens. Treatment 1, control; treatment 2, *E. coli* challenge at 10^3 cfu/mL; treatment 3, *E. coli* challenge at 10^3 cfu/mL mixed with 10^3 pfu/mL of bacteriophage; treatment 4, *E. coli* challenge at 10^3 cfu/mL mixed with 10^6 pfu/mL of bacteriophage; treatment 5, *E. coli* challenge at 10^4 cfu/mL; treatment 6, *E. coli* challenge at 10^4 cfu/mL mixed with 10^4 pfu/mL of bacteriophage; and treatment 7, *E. coli* challenge at 10^4 mixed with 10^8 pfu/mL of bacteriophage. [a–c]Vertical bars with different letters differ significantly ($P \leq 0.05$).

and DAF6, were selected for these studies based on size and clarity of plaques. They were amplified by inoculating a culture of *E. coli* grown for 2.5 h with bacteriophage followed by overnight incubation at 37°C. The cultures were then centrifuged (2,500 × *g*) and passed through a 0.2-μm membrane filter. Bacteriophage enumeration was conducted using the soft agar overlay procedures as described in detail by Huff et al. (2002b).

Our initial study was conducted to determine if mixing our *E. coli* challenge culture with a single bacteriophage prior to challenging the birds would prevent the onset of colibacillosis (Huff et al., 2002b). At 7 d of age birds were challenged by injecting *E. coli* in the air sac at 10^3 cfu/mL or with the challenge culture mixed with bacteriophage SPR02 at 10^3 or 10^6 pfu/mL. In addition, birds were challenged via air sac with *E. coli* at 10^4 cfu/mL or with the challenge culture mixed with bacteriophage SPR02 at 10^4 or 10^8 pfu/mL. These studies were concluded when the birds were 3 wk of age. The effects of these treatments on mortality are presented in Figure 1. Mortality was significantly decreased from 80% (treatment 1) in the birds that received 10^3 cfu/mL of *E. coli* to 25 and 5% when the challenge culture was mixed with 10^3 or 10^6 pfu/mL of the bacteriophage, respectively (treatments 3 and 4). Mortality was significantly decreased from 85% (treatment 5) in birds that received 10^4 cfu/mL of *E. coli* to 35% in the birds challenged with 10^4 cfu/mL of *E. coli* mixed with 10^4 pfu/mL of bacteriophage (treatment 6). There was complete protection when this challenge culture was mixed with 10^8 pfu/mL of the bacteriophage (treatment 7). Although these initial studies represented a somewhat artificial experimental design, they demonstrate that if bacteriophage are present at the site of the bacterial infection at high enough titers, the bacteriophage

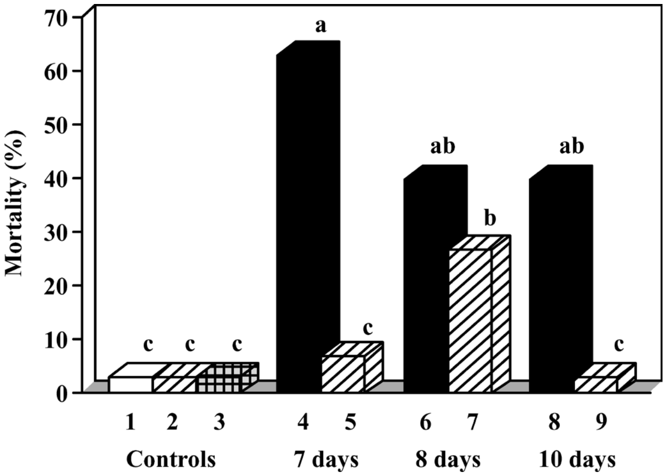

FIGURE 2. The effect on mortality (%) after administration of an aerosol spray of bacteriophage before challenging the bird with *E. coli* to prevent severe colibacillosis in broiler chickens. Treatment 1, control; treatment 2, control spray; treatment 3, bacteriophage spray; treatment 4, control spray at 7 d of age challenged with *E. coli* at 7 d of age; treatment 5, bacteriophage spray at 7 d of age challenged with *E. coli* at 7 d of age; treatment 6, control spray at 7 d of age challenged with *E. coli* at 8 d of age; treatment 7, bacteriophage spray at 7 d of age challenged with *E. coli* at 8 d of age; treatment 8, control spray at 7 d of age challenged with *E. coli* at 10 d of age; and treatment 9, bacteriophage spray at 7 d of age challenged with *E. coli* at 10 d of age. [a–c]Vertical bars with different letters differ significantly ($P \leq 0.05$).

are effective in preventing the bacterial infection, in this case, the onset of severe colibacillosis.

Bacteriophage might be used to prevent bacterial infections. To investigate the potential of bacteriophage to prevent bacterial infections, we conducted several studies to determine if bacteriophage could prevent colibacillosis (Huff et al., 2002a). The basic experimental design was to administer the bacteriophage as an aerosol spray prior to challenging the birds with *E. coli.* In these studies we sprayed the birds with a diluent (PBS) or bacteriophage cocktail containing bacteriophage SPR02 (10^8 pfu/mL) and DAF6 (10^9 pfu/mL) when the birds were 7 d of age. The birds were then challenged with *E. coli* by air sac inoculation with10^4 cfu/mL immediately after being sprayed with the diluent or bacteriophage (7 d of age) or at 8 or 10 d of age. These and subsequent data were presented as percentages and transformed as the square root of the arc sine prior to statistical analysis. Pen means were the unit for statistical analysis. Significant differences between treatments were separated using least squares means procedures of SAS software. All statements of significance are based on a probability level of 0.05.The effects of these treatments on mortality are presented in Figure 2. The birds that were sprayed with PBS and immediately challenged with *E. col*i had 63% mortality (treatment 4), whereas only 7% of birds died when sprayed with the bacteriophage cocktail and immediately challenged with *E. coil* (treatment 5). Mortality of birds sprayed only with PBS at 7 d of age and challenged a day later (8 d of age) was 40% (treatment 6). Mortality of birds sprayed with 2 bacteriophages at 7 d of age and challenged with *E. coli* 1 d later (8 d of age) was 27% (treatment 7), which was not significantly different from birds sprayed with PBS and challenged at 8 d of age. Mortality of birds sprayed with PBS at 7 d of age and challenged with *E. coli* 3 d later (10 d of age) was 40% (treatment 8). Mortality of birds sprayed with 2 bacteriophages at 7 d of age and challenged with *E. coli* at 10 d of age was 3% (treatment 9), which was significantly less than the mortality observed for birds sprayed with PBS at 7 d of age and challenged with *E. coli* at 10 d of age (40%). In these studies bacteriophage were effective at preventing the onset of severe colibacillosis when administered immediately prior to challenging the birds with *E. coli*, and in the trial presented here was effective in preventing the onset of severe colibacillosis for up to 3 d after the bacteriophages were administered. These data have practical significance given that aerosol sprays are, and have been, used by the poultry industry in the hatchery and grow-out environments. It might be possible to reduce the incidence of colibacillosis in poultry production by spraying the birds with bacteriophage in the hatchery, which would provide some protection to the chicks for some time after placement when they are the most susceptible to respiratory infections. In addition, it may be possible and practical to spray flocks of older birds experiencing an acute outbreak of colibacillosis to protect the birds in the flock from horizontal infection from their acutely infected flock mates.

We have continued our work with bacteriophage to determine the efficacy of bacteriophage to treat bacterial infections. The general design of these studies has been to challenge birds with *E. coli* followed with bacteriophage treatment as an aerosol spray or an intramuscular injection of bacteriophage (Huff et al., 2003a). Although aerosol spray provides good protection of the birds from the onset of severe colibacillosis, it is not very effective in treating colibacillosis once the infection become systemic. However, an intramuscular injection of bacteriophage is a very effective treatment of colibacillosis as shown in Figure 3. In these studies birds were challenged with E. coli with an air sac inoculation when they were 7 d of age, which was followed by an intramuscular injection of bacteriophage immediately after challenge (7 d of age), 24 h after challenge (8 d of age), or 48 h after challenge (9 d of age). Significant treatment efficacy to rescue the birds from severe colibacillosis was observed even when treatment was delayed for 48 h after challenge. An intramuscular injection of bacteriophage produces rapid and relatively high titers of systemic bacteriophage compared with the systemic levels of bacteriophage that can be obtained with aerosol administration of bacteriophage (Huff et al., 2003a). This difference in the blood levels of bacteriophage makes a big difference in the therapeutic efficacy of intramuscular injection of bacteriophage compared to aerosol administration of bacteriophage. Once the birds are air sac challenged with *E. coli*, the infection becomes systemic, very quickly requiring systemic levels of any therapeutic agent, in this case bacteriophage. We have also found that multiple intramuscular injections of bacteriophage provide better therapeutic efficacy then a

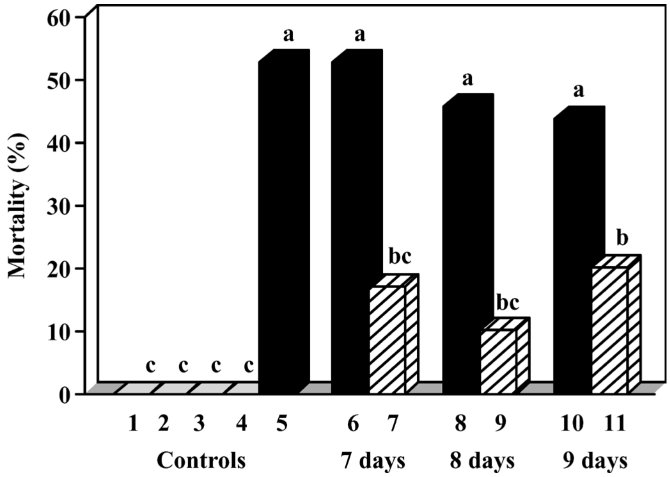

FIGURE 3. The effect on mortality (%) of an intramuscular injection of bacteriophage after challenging the bird with *Escherichia coli* to treat severe colibacillosis in broiler chickens. Treatment 1, control; treatment 2, PBS challenge; treatment 3, heat-killed bacteriophage injection; treatment 4, active bacteriophage injection; treatment 5, *E. coli* challenged at 7 d of age; treatment 6, *E. coli* challenged at 7 d of age heat killed bacteriophage injection at 7 d of age; treatment 7, *E. coli* challenged at 7 d of age bacteriophage injection at 7 d of age; treatment 8, *E. coli* challenged at 7 d of age heat killed bacteriophage injection at 8 d of age; treatment 9, *E. coli* challenged at 7 d of age bacteriophage injection at 8 d of age; treatment 10, *E. coli* challenged at 7 d of age heat killed bacteriophage injection at 9 d of age; and treatment 11, *E. coli* challenged at 7 d of age bacteriophage injection at 9 d of age. [a–c]Vertical bars with different letters differ significantly ($P \leq 0.05$).

single intramuscular injection of bacteriophage (Huff et al., 2003b).

SUMMARY

The potential of bacteriophage to reduce the impact of bacterial pathogens in a variety of applications appears great given the fact that bacteriophage kill bacteria. Our work and that of many others, cited above, has documented the potential of bacteriophage to be an effective alternative to antibiotics. There are some perceived and real limitations of the potential of bacteriophage that have affected their acceptance and practicality, which we have discussed in detail in an earlier publication (Huff et al., 2004). Some of these limitations include their specificity, their ability to act as a vehicle of bacterial genes with consequent transformation of infected bacteria, uncertainty over regulatory acceptance of bacteriophage, concern over protection of proprietary rights of bacteriophage products, fragile activity, and practical routes of administration of bacteriophage in animal production systems. Many, if not all, of these limitations can be solved by research. The concept that bacteriophage can be used in several ways to decrease the negative impact of bacterial pathogens on poultry production has been demonstrated, and bacteriophage can provide an effective alternative to antibiotics. The challenge is not whether bacteriophage work, but which bacteria pathogens should we target for bacteriophage control, and how can we make bacteriophage practical within our poultry production systems.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Scott Zornes, Dana Bassi, David Horlick, Sonia Tsai, and Wally McDonner.

REFERENCES

Alisky, J., K. Iczkowski, A. Rapoport, and N. Troitsky. 1998. Bacteriophages show promise as antimicrobial agents. J. Infect. 36:5–15.

Barrow, P., M. Lovell, and A. Berchieri, Jr. 1998. Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and meningitis in chickens and calves. Clin. Diagn. Lab. Immunol. 5:294–298.

Biswas, B, S. Adhya, P. Washart, B. Paul, A. N. Trostel, B. Powell, R. Carlton, and C. R. Merril. 2002. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. Infect. Immun. 70:204–210.

Cerveny, K. E., A. DePaola, D. H. Duckworth, and P. A. Gulig. 2002. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. Infect. Immun. 70:6251–6262.

d'Herelle, F. 1917. Sur un microbe invisible antagonists des *bacilles dysenteriques*. C. R. Acad. Sci. Paris 165:373–375.

Goode, D. H., V. M. Allen, and P. A. Barrow. 2003. Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophage. Appl. Environ. Microbiol. 69:5032–5036.

Higgins, J. P., S. E. Higgins, K. L. Guenther, W. E. Huff, and B. M. Hargis. 2002. Evaluation of bacteriophage treatment as a method to reduce culturable *Salmonella* in poultry carcass rinse water. Poult. Sci. 81(Suppl. 1):130. (Abstr.)

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue. 2002a. Prevention of *Escherichia coli* infection in broiler chickens with a bacteriophage aerosol spray. Poult. Sci. 81:1486–1491.

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue. 2003a. Evaluation of aerosol spray and intramuscular injection of bacteriophage to treat an *Escherichia coli* respiratory infection. Poult. Sci. 82:1108–1112.

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue. 2003b. Bacteriophage treatment of a severe *Escherichia coli* respiratory infection in broiler chickens. Avian Dis. 47:1399–1405.

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue. 2004. Bacteriophage: Potential role in food safety. Pages 365–374 in Preharvest and postharvest Food Safety Contemporary Issues and Future Directions. R. C. Beier, S. D. Pillai, T. D. Phillips, R. L Ziprin, ed. Blackwell Publishing, Ames, IA.

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, H. Xie, P. A. Moore, Jr., and A. M. Donoghue. 2002b. Prevention of *Escherichia coli* respiratory infection in broiler chickens with bacteriophage (SPR02). Poult. Sci. 81:437–441.

Leverentz, B., W. S. Conway, M. J. Camp, W. J. Janisiewicz, T. Abuladze, M. Yang, R. Saftner, and A. Sulakvelidze. 2003. Biocontrol of *Listeria moncytogenes* on fresh-cut produce by treatment with lytic bacteriophage and a bacteriocin. Appl. Environ. Microbiol. 69:4519–4526.

Matsuzaki, S, M. Yasuda, H. Nishikawa, M. Kuroda, T. Ujihara, T. Shuin, Y. Shen, Z. Jin, S. Fujimoto, M. D. Nasimuzzaman, H. Wakiguchi, S. Sugihara, T. Sugiura, S. Koda, A. Muraoka, and S. Imai. 2003. Experimental protection of mice against lethal *Staphylococcus aureus* infection by novel bacteriophage ∅MR11. J. Infect. Dis. 187:613–624.

Park, S. C., I. Shimamura, M. Fukunaga, K. Mori, and T. Nakai. 2000. Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. Appl. Environ. Microbiol. 66:1416–1422.

Ślopek, S., I. Durlakowa, B. Weber-Dąbrowska, M. Dąbrowski, and A. Kucharewicz-Krukowska. 1984. Results of bacteriophage treatment of suppurative bacterial infections: III. Detailed evaluation of the results obtained in further 150 cases. Arch. Immunol. Ther. Exp. 32:317–335.

Ślopek, S., I. Durlakowa, B. Weber-Dąbrowska, M. Dąbrowski, and A. Kucharewicz-Krukowska. 1987. Results of bacteriophage treatment of suppurative bacterial infections in the years 1981–1986. Arch. Immunol. Ther. Exp. 35:569–583.

Ślopek, S., I. Durlakowa, B. Weber-Dąbrowska, A. Kucharewicz-Krukowska, M. Dábrowski, and R. Bisikiewicz. 1981. Results of bacteriophage treatment of suppurative bacterial infections: II. Detailed evaluation of the results. Arch. Immunol. Ther. Exp. 31:293–327.

Ślopek, S., A. Kucharewicz-Krukowska, B. Weber-Dąbrowska, and M. Dąbrowski. 1985. Results of bactiophage treatment of suppurative bacterial infections. VI. Analysis of treatment of suppurative staphylococcal infections. Arch. Immunol. Ther. Exp. 33:261–273.

Smith, H. W., and M. B. Huggins. 1982. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. J. Gen. Microbiol. 128:307–318.

Smith, H. W., and M. B. Huggins. 1983. Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. J. Gen. Microbiol. 129:2659–2675.

Smith, H. W., M. B. Huggins, and K. M. Shaw. 1987. The control of experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. J. Gen. Microbiol. 133:1111–1126.

Soothill, J. S. 1992. Treatment of experimental infections of mice with bacteriophages. J. Med. Microbiol. 37:258–261.

Twort, F. W. 1915. An investigation on the nature of ultramicroscopic viruses. Lancet 2:1241–1243.

Weber-Dąbrowska, B., M. Dąbrowski, and S. Ślopek. 1987. Studies on bacteriophage penetration in patients subjected to phage therapy. Arch. Immunol. Ther. Exp. 35:563–568.